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Induced by Chronic Exposure to Depleted Uranium

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14. ABSTRACT The chemical properties and high density of depleted uranium (DU) render the metal well suited for military purposes, but knowledge of DU neurotoxicity and its treatment is lacking. This project is designed to test the hypothesis that long-term administration of an anti-oxidant agent and/or an NMDA receptor antagonist will reduce neurotoxicity resulting from chronic exposure to DU. This hypothesis is based on previous observations in rats chronically exposed to DU, and reflects the anticipation that specific pharmacological agents will reverse signs of DU-induced oxidative stress. As prescribed by the Statement of Work, efforts continued in year 2 on Tasks 1 (drug therapies to reverse DU-induced neurotoxicity) and 2 (brain DU concentrations) utilizing experimental groups (0, 300, and 600 mg DU) exposed for 9 months. Task 1 is complete, but the responses of DU-exposed animals to the drugs did not exhibit neuroprotective potential. Progress has been achieved on Tasks 2-4, and testing of remaining subject cohorts is being completed in the no cost extension period. Thus, progress is proceeding according to the Statement of Work and will be completed during the additional interval.					
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INTRODUCTION

The chemical properties and high density of depleted uranium (DU) render the metal well suited for military purposes. The U. S. Army utilizes DU for tank armor and in munitions, deployed such weapons in the first Gulf War, and is currently deploying them in Afghanistan. However, knowledge of DU neurotoxicity and its treatment is lacking despite reports of exposed cohorts exhibiting neurocognitive dysfunction (1). Research in chronically exposed rats has reported alterations in hippocampal synaptic transmission, suggesting DU-induced decreases in neuronal excitability (2). This project examines potential treatment options to address neurotoxicity from chronic DU exposure. On the basis of previous observations the bases of DU neurotoxicity are proposed to be cellular oxidative stress and the consequent increased production of reactive oxygen species, leading to decreased glutamate uptake and increased synaptic glutamate concentrations in conjunction with NMDA receptor up-regulation. Uranium-induced oxidative stress has previously been reported in rat kidney, testis, and lung (3-4). Studies will identify various biochemical markers of metal-induced oxidative stress in hippocampal and cortical tissue, and in combination with enhanced extracellular glutamate and NMDA receptor activity will provide three components of DU neurotoxicity for assessment of therapeutic efficacy. *It is hypothesized that long-term administration of an anti-excitotoxic agent and/or an NMDA receptor antagonist will reduce DU neurotoxicity.* These studies will provide critical information on which to base new treatments for exposed Gulf War veterans.

BODY

As prescribed in the Statement of Work, project activities in year 3 primarily addressed completion of Task 1, but also involved significant progress in the performance of Tasks 3 and 4. A description of these efforts and the resulting progress toward completion of each objective is provided below.

Task 1 concerned demonstration of the efficacy of chronically administered drug therapies to reverse DU-induced elevations in extracellular glutamate in superfused hippocampal slices from chronically exposed animals. The project included a control group and low (300 mg load) and high dose (600 mg load) DU exposure conditions, but utilized a vehicle and three drug-treated groups (memantine or riluzole or a combination) for each exposure level. This design resulted in a 3 exposure level \times 4 drug condition matrix with ~8 animals/cell, thus maximizing the ability to discriminate the actions of the therapeutic agents on the proposed measures. Drugs were administered via osmotic minipumps (Alzet) surgically inserted subcutaneously. Adult male Sprague-Dawley rats were implanted intramuscularly with DU pellets at 70-80 days of age; beginning at 3-4 months of age they were placed on food restriction so that their maximal body weight did not exceed 500-550 grams. After 7 months exposure 28-day minipumps were implanted and replaced once to cover the period up to 9 months when exposure was terminated and testing conducted. This is an appropriate interval for drug administration as this regimen simulates DU exposure during the first Gulf War and potential treatments applied long after exposure was initiated. The minipumps were filled with drug solutions of 30 mg/ml memantine (3.6 mg/kg/day dose) and/or 10 mg/ml riluzole (1.2 mg/kg/day dose). Besides its potential usefulness as an uncompetitive NMDA receptor antagonist, memantine also has been reported to have neuroprotectant value via induction of brain-derived neurotrophic factor and its receptor (5-7), making the drug of particular interest for this project. Blood samples were collected from sufficient animals prior to sacrifice to establish the plasma drug levels achieved and validate the drug administration

protocols. The study described in Task 1 is now complete. Table 1 lists the plasma drug levels achieved over a period of 7-8 weeks with this dosing regimen.

Plasma Drug Levels, ng/ml		
Group	MEM	RIL
Vehicle	- 0	-- 0
Memantine	28.1 ± 3.4	--
Riluzole	--	41.1 ± 3.0
Memantine & Riluzole	17.2 ± 1.2	24.5 ± 2.3
Values are mean ± SEM, N = 8-9. Blood sampled from jugular vein 7-8 weeks after drugs instituted. MEM = memantine; RIL = riluzole.		

Evidence from previous work has indicated that the effects of chronic exposure to DU on depolarization-induced hippocampal extracellular glutamate are a combination of acute UO_2^{+2} -dependent inhibition of release (8) and an opposing slowly developing increase in extracellular transmitter (9), perhaps due to metal-induced oxidative stress leading to mitochondrial and glutamate transporter dysfunction. An additional component of the observed neurotoxic response is a substantial up-regulation of NMDA receptor density. Data from the study addressing Task 1 are shown in Figures 1-3. The results display depolarization-induced glutamate release from K^+ -stimulated

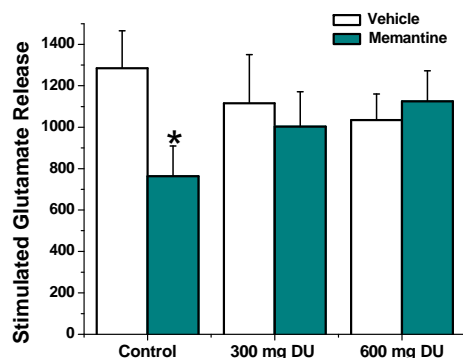


Figure 1. Evoked glutamate release from K^+ -stimulated hippocampal slices after exposure to DU for 9 months and continual administration of memantine for months 8-9 via osmotic minipumps. Values are expressed as mean ± SEM (N = 7/group) of the area under the curve normalized to 1.0 and summed across the peak response intervals. * $p < 0.05$ compared to the paired group receiving vehicle only in the minipumps.

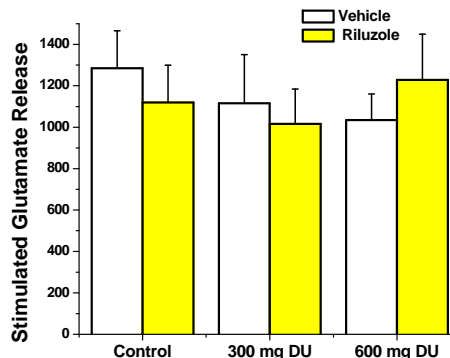


Figure 2. Evoked glutamate release from K^+ -stimulated hippocampal slices after exposure to DU for 9 months and continual administration of riluzole for months 8-9 via osmotic minipumps. Values are expressed as mean ± SEM (N = 6-7/group) of the area under the curve normalized to 1.0 and summed across the peak response intervals.

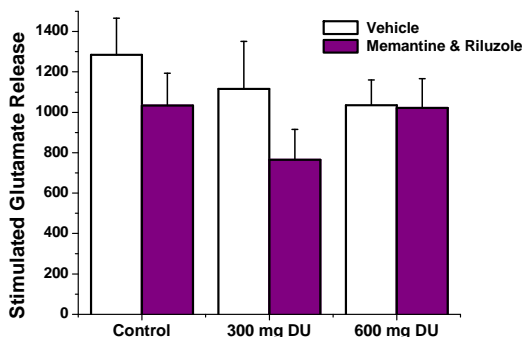


Figure 3. Evoked glutamate release from K^+ -stimulated hippocampal slices after exposure to DU for 9 months and continual administration of memantine and riluzole for months 8-9 via osmotic minipumps. Values are expressed as mean ± SEM (N = 6-7/group) of the area under the curve normalized to 1.0 and summed across the peak response intervals.

hippocampal slices after exposure to DU for 9 months in conjunction with continual administration of memantine (Figure 1), riluzole (Figure 2), or the combination (Figure 3) for months 8-9 via osmotic minipumps.

In addition, independent control and DU groups were generated that did not receive minipumps containing vehicle, and thus more closely simulated the exposure of study groups in the earlier project. As a result of these data two observations are apparent. First, the enhancement of evoked extracellular glutamate concentrations found after 14-17 months of DU exposure in earlier work was not discriminated after 9 months of exposure (Figure 4), suggesting that longer periods of administration are necessary for the glutamate transporter dysfunction to become the dominant effect over the opposing UO_2^{+2} -dependent inhibition of release. Second, memantine significantly diminished evoked extracellular glutamate in control vehicle-treated animals, but had no effect on groups chronically exposed to DU. Administration of riluzole and the memantine/riluzole combination did not alter glutamate responses to high K^+ in any groups.

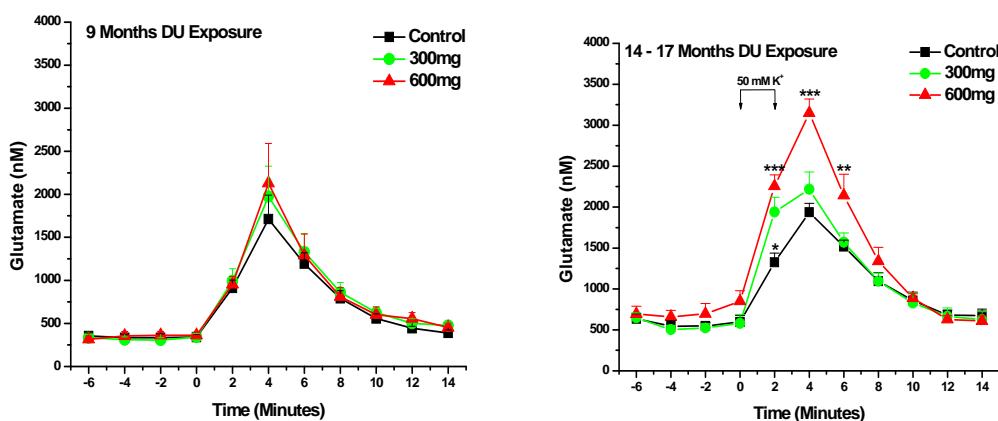


Figure 4. Time course of glutamate concentration in response to superfusion with 50 mM K^+ across transverse hippocampal slices in 10 mM HEPES-sucrose buffer (pH 7.4). The stimulus-evoked increase in endogenous glutamate was significantly enhanced by exposure to 300 or 600 mg implanted DU over 14-17 months (*Right*), but not at 9 months (*Left*). Values are expressed as mean \pm SEM based on $N = 8$ /group with sample determinations conducted in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ relative to the glutamate concentration in control animals at the same time point.

The bases for the attenuation of stimulated glutamate release by memantine in control animals, and the absence of this effect in the DU-exposed groups requires further investigation. Since memantine is an antagonist at NMDA receptors, the DU-induced receptor up-regulation may result in a smaller proportion of inhibited NMDA receptors in the exposed than in the control groups, and thereby cause reduced drug effectiveness in DU animals. However, memantine also exhibits other pharmacological actions that must be considered, such as inhibiting nicotinic receptors (10) and enhancing neurotrophic factor function (e.g., 7).

It is possible that the doses of memantine/riluzole utilized were not adequate to produce more consistent effects in the control and DU groups. If this were true, another route of administration likely would have to be chosen, since the concentrations of drug applied through the osmotic minipumps approached their solubility limits. A longer duration of drug administration may also have

been more effective in countering DU neurotoxicity, but such a regimen would have less relevance to the Gulf War cohort of exposed soldiers who remain essentially untreated at this time.

Task 2 consisted of determination of DU concentrations in brain tissue of chronically exposed animals at durations corresponding to the beginning (7 months) and end (9 months) of the drug therapies. Characterization of the protocol in this Task was designed to provide context to the experimental findings generated under the other Tasks. The determination of hippocampal uranium levels would be performed by inductively coupled plasma-mass spectrometry (ICP-MS) analysis by a commercial laboratory. This methodology has proven more sensitive and reliable for this sample matrix than alternative approaches. The DU used in this project consists of 30 mg pellets (1 mm diameter \times 2 mm length) obtained from Aerojet Ordnance Tennessee (Jonesborough, TN), and were sterilized prior to use. Ten pellets were implanted in the gastrocnemius muscle of each thigh of 70-80 day old male rats. The design included three exposure groups: a high dose group in which all pellets were DU (600 mg load), a low dose group receiving 10 pellets of DU (300 mg load), and a control group which received 20 tantalum pellets (0 mg load). The low dose group also received 10 pellets of tantalum. Tantalum is an essentially inert heavy metal widely used in medical prostheses. The group size (N = 6) was sufficient to characterize the exposure protocol and provide general measures of metal uptake. At this point all tissue samples for these analyses have been harvested and are awaiting shipment to the analytical laboratory. However, the tissue analyses for DU to complete Task 2 (cost of ~\$10K) have been delayed until funds could be identified to cover these expenses. Because of the thorough characterization of the exposure regimen in earlier work (9) and the cost of other unforeseen expenses (see below), these analyses have not been performed at this time.

In order to maximize the efficiency of use of the last cohort of animals Tasks 3 and 4 were performed on brain tissue harvested from this same group of rats. Task 3 concerned assessments of biochemical markers of DU-induced oxidative stress in brain tissue and the ability of drug therapies to reverse the changes in these measures. Task 4 quantified DU-induced elevations in NMDA receptor binding density and the ability of drug therapies to reverse these measures in tissue membrane preparations using radioligand binding. Brains were harvested and 400 μ m transverse slices were cut in a vibratome at 4°C from a midbrain block of tissue in 10 mM HEPES-sucrose buffer (pH 7.4). Parietal cortical slices were used for the glutamate uptake assay and the associated hippocampal slices pooled for NMDA receptor binding. Cerebellum, striatum, and frontal cortex were also dissected and stored for assessing levels of oxidative stress related to DU neurotoxicity. DU exposure in this cohort of animals is nearing an end, with only replacement animals to complete various treatment groups still undergoing the regimen. The determination of biochemical markers utilized commercially available kits and assessed catalase and glutathione peroxidase activities. Data collection on these samples is currently being performed. ³H-Glutamate uptake was measured in cortical slices as a focused assessment of the integrity of the neurotransmitter transport process. It is well known that induced oxidative stress results in mitochondrial dysfunction and decreased energy production, leading to reduced glutamate transporter activity. These data are currently undergoing statistical analysis. NMDA receptor binding experiments are also currently being conducted, and early results indicate that the previously reported DU-induced up-regulation in receptor density of this glutamatergic receptor subtype after 14-17 months exposure is also evident after the shorter 9 month exposure interval.

Unavoidable delays have occurred in the completion of project Tasks in year 3. One member of the project lab staff left the University at the end of July, 2009. A qualified postdoctoral associate was

identified to quickly fill this role, but could not accept the position due to visa problems. A new search for a laboratory technician was initiated, and the candidate finally began work on the project in December, 2009. These problems delayed progress of some of the experimentation. In addition, some difficulties have been encountered with the surgical implantation and function of the osmotic minipumps responsible for therapeutic drug administration to the DU-exposed animals in the project. Accordingly, a number of experimental animals had to be replaced in the studies to insure that reliable data were obtained, and the experimental treatment of the last of these replacement animals will not be completed until November, 2010.

Cost overruns have posed continuing problems throughout the project. These charges were primarily traceable to the higher costs of DU pellets than in previous work, the high expense of the osmotic minipumps which could not be refilled over the period of drug administration but had to be replaced, and the unbudgeted charges for having plasma drug levels determined by an analytical laboratory. The latter analyses were necessary to establish the plasma drug levels achieved and to validate the drug administration protocols. The tissue analyses for DU to complete Task 2 (cost of ~\$10K) have been delayed until funds could be identified to cover these expenses. Otherwise, the remaining aspects of the Statement of Work will be completed by the end of the no cost extension period.

KEY RESEARCH ACCOMPLISHMENTS

Considerable effort has been invested to optimize the surgical procedures for DU pellet and osmotic minipump implants, particularly since the latter had to be replaced once in each animal at the 8 month exposure interval. The consistency and reliability of the drug administration regimen is critically important in being able to demonstrate a neuroprotective/antioxidant effect for memantine and/or riluzole, and plasma drug determinations support the other neurochemical tests being conducted.

Performance of the complex experimental design involved in Task 1 is complete, but there is no evidence at this point that memantine and/or riluzole are effective in the treatment of this aspect of DU neurotoxicity. Memantine was effective in reducing evoked hippocampal glutamate release in the control (i.e., tantalum) group, but no effect was observed in the DU-exposed groups (Figure 1). Completion of the other Tasks may be more elucidating.

REPORTABLE OUTCOMES

Task 1 is completed at this time. Preliminary reports of the work performed on Task 1 have been reported in abstract form at the 2009 Military Health Research Forum (August 31 - September 3) and 2009 Society for Neuroscience (October 17-21) meetings. Tasks 3 and 4 are nearing completion, but data collection is ongoing, and there are no further outcomes to report at this time.

CONCLUSIONS

Summaries of progress on the project and its importance as a scientific product are included in the preceding sections **Body** and also in **Key Research Accomplishments**. General conclusions on the effectiveness of memantine and/or riluzole to reverse the effects of DU-induced neurotoxicity cannot be stated with finality at this time, as the drugs may prove useful in reversing the measures of DU neurotoxicity currently under investigation in Tasks 3 and 4. The chronic DU exposure and drug

administration protocols have been established by analytical determinations conducted during years 2 and 3 of the project.

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APPENDICES

None

SUPPORTING DATA

None